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Research Article

IN VIVO ASSESSMENT OF WOUND CLOSURE AND BIOCOMPATIBILITY OF NANOCHITOSAN-POLYVINYLALCOHOL-GLYCEROL FILMS IN EXPERIMENTAL ANIMAL MODEL

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ABSTRACT

The need for innovative wound dressings has arisen due to injuries from second intention healing and their management is associated with high costs. Modern dressings not only protect wounds but also promote proliferation of cells and tissue regeneration. Patient compliance is crucial, ensuring that the dressing removal does not harm the newly regenerated tissue. In this study, nano-chitosan polyvinyl alcohol- glycerol (NCH-PVA-GLY) transparent wound dressing was developed that expedites tissue repair and can be removed through an external stimulus without disrupting the regenerated tissue. This dressing efficiently absorbs excess wound exudates while maintaining proper hydration. Evaluation in male Wistar rats with wounds revealed that the dressing, when applied for 16 days, resulted in minimal inflammation and denser connective tissue compared to wounds devoid of dressings. The dressing exhibited enhanced re-epithelization and angiogenesis, facilitating effective wound closure. The exposure of the resultant dressing, to the incised wound facilitated complete wound closure in 16 days, suggesting the effectiveness of the dressings to cure superficial wound.

Keywords: Nanochitosan, Polyvinyl alcohol, Glycerol, Dressing films**,** Wound healing.

INTRODUCTION

Wound healing stands as an intricate biological phenomenon, where numerous factors, including the wound's condition, the overall health of the patient, and the reinforcement of external materials with specific physicochemical attributes, play critical roles (Moeini *et al*., 2020). The advancements in wound care management pivot on the innovation of bioactive compounds and engineered tissue substitutes, vital for expediting the intricate process of healing. The exploration of the marine environment has emerged as a key factor in unveiling natural products housing molecules conducive to effective wound healing, as evidenced by prior research (Chandika *et al*., 2015; Amruth *et al*., 2021).

The foremost objective of wound care is to hasten the healing process, prevent infections, alleviate pain and discomfort, and minimize scarring. Achieving successful wound treatment involves the reduction of necrotic tissue, prevention of microbial intrusion, and the creation of optimal conditions favourable to successful healing outcomes, a focus underscored in recent studies (Aramwit, 2016). Various wound dressings, including films, foams, hydrogels, hydrocolloids and hydrofibers, play pivotal roles in realizing these objectives. Among these, film-based dressings stand out, offering a spectrum of advantages such as gas permeability, impermeability to liquid and bacteria, direct drug delivery capabilities, flexibility, moisture control, pain reduction, a barrier against contamination, and ease of wound bed inspection without the removal of

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dressing (Savencu *et al.*, 2021; Amruth *et al.*, 2021 and 2022).

The significant burden of chronic wounds exists globally, demanding substantial financial resources for effective management, estimated to reach 1000 USD per treatment (West and Jones, 2007). Chronic wounds, encompassing conditions like complex burns, diabetes-related wounds, venous ulcers, and pressure ulcers pose challenges due to their prolonged healing duration compared to other wound types (Izadi and Ganchi, 2005; Ignacio *et al*., 2011). Wounds with the potential for healing through secondary intention are prevalent, often characterized by the presence of visible granulation tissue(McCaughan *et al*., 2018).Numerous medical dressings have been developed for wound protection and healing considering its widespread use, financial implications, and profound effect on individuals(Cho *et al*., 1999, Smith *et al.,* 2016).These effectively safeguard the injured site and expedite closure of the wound, but sometimes may induce attachment between the newly generated tissue and the dressing upon application, leading to complications during dressing removal (Edahiro *et al*., 2005).

Chitosan derived from crustaceans, emerged as natural polymer for wound dressings because of its diverse beneficial properties like the ability to form films, nontoxicity, low immunogenicity, antibacterial, antifungal, hemostatic, and muco adhesive characteristics (Chen *et al*., 2020). The versatility of chitosan extends to its biocompatibility, biodegradability, and efficiency in controlling drug release, rendering it a promising material. Since chitosan has been converted into nanochitosan, it has the advantage of having smaller particle sizes, which may allow for better penetration of the wound region and across skin tissue for more effective wound healing effects. Due to the polymer's composition, most of the particles in nanochitosan have a significant positive charge, and because like charges repel one another to prevent aggregation, the positive charge of the particles offers a special set of advantages. Strong surface positive charges on the nanoparticles enable them to interact more easily with a variety of negatively charged materials, including bacteria cells, mucosal surfaces, and cell surfaces (Loo *et al*., 2022). The mechanical strength of natural polymers are enhanced through cross-linking or blending with synthetic polymers by maintaining biocompatibility (Mogosanu and Grumezescu, 2014).Polyvinyl alcohol (PVA), a widely used water-soluble synthetic polymer, contributes to stable film formation with favorable mechanical characteristics. Strategies such as covalent cross-linking and the addition of plasticizers, such as glycerol, further enhance PVA film flexibility (Srinivasa *et al.*, 2003, Savencu *et al*., 2021).

The study aims to the understanding of wound closure rates in incised areas by evaluating the application of a nanochitosan-polyvinyl alcoholglycerol film (NCH-PVA-GLY) dressing. The investigation aligns with the broader objective of advancing wound care strategies and optimizing the use of biomaterials for improved therapeutic outcomes.

MATERIALS AND METHODS

Materials

Low molecular weight Chitosan with deacetylation ≥ 75 % and Poly vinyl alcohol (PVA) were purchased from Sigma-Aldrich Chemical Pvt Limited, Bangalore, India. All of the chemicals and reagents utilised in this investigation, including glycerol, were of guaranteed grade (GR) or analytical grade (AR).

Preparation of Nano chitosan

A solution of 1% chitosan was utilized in the creation of nanochitosan through the gradual addition of sodium tripolyphosphate (STPP). The solution placed on a magnetic stirrer, was stirred for 1 hour. Subsequently, the solution underwent ultrasonication to generate nanochitosan (Rampino *et al*., 2013).To the resulting nanochitosan solution, 5% polyvinyl alcohol (PVA) were added while continuously stirring till a clear solution was achieved. The dissolved solution was then poured onto a tray and left overnight in a hot air oven at 55°C to obtain the nanochitosan film (Bahrami, *et al*., 2015)

In vivo wound healing and biocompatibility study

The nanochitosan films (Experimental control) were employed in a pocket wound model made in albino male Wistar rats. Before the commencement of the study, the animals with a mean body weight of 175-200g aged 8 weeks underwent 7-day acclimatization. Under standard environmental conditions of temperature 28 ± 2°C, humidity ranging from 60-70%, animals were housed in polypropylene cages, adhering to a 12-hour light/dark cycle. The rats were fed a normal diet and were allowed unlimited access to water.

The design of experiment comprised three groups, each consisting of six rats. The rats in the I^{st} group served as the control group where the animals were left with an open wound. The group II received treatment with the transparent film dressing, NCH-PVA-GLY whereas the third group was treated with a commercial dressing. The experiment initiated with the intraperitoneal infusion of ketamine (75 mg/kg body weight) and xylazine (10 mg/kg body weight) to the animals, which made them unconscious. The rats were shaved at the dorsal portion and sterilized using a 70% ethanol solution. An approximately 15 mm incision was created using a sterilized surgical blade, forming a pouch subcutaneouslyon one side of the incision. A dressing material measuring around 1 mm was inserted into the pocket and sutured with a nonabsorbable silk thread. The animals were observed and the wounds were measured up to 16^{th} day. The rats were euthanized at 28 days and 60 days and skin tissues with the surrounding implant area were collected for histopathological analysis

(Sumayya and Muraleedhara Kurup, 2018; Amruth *et al*., 2023). The Animal Ethics Committee, ICAR-Central Institute of Fisheries Technology, Cochin, Kerala, India, provided the ethical approval number (CIFT/B&N/IAEC/2021-1(2)a) for the research, and in order to protect the welfare of the animals, the study complied with all IAEC guidelines.

Measurement of wound closure

Throughout the duration of the study, the wounds treated with different dressings were carefully inspected to evaluate dressing integrity and monitor animal behaviour. Using an iPhone 10 XR, prone photos of every animal were taken so that scars could be assessed and group comparisons could be made. Each wound on the six animal groups was evaluated and photographed three times at predetermined intervals, namely on days 0, 3, 7, 12, and 16. The closure rate of the wound was calculated with the following equation:

$$\text{Wound closure rate}\,(\%) = \frac{Wo - Wt}{Wo} * 100$$

Wo signifies wound area measured initially

Wt represents the area of wound at a particular time "t".

This formulation facilitates the estimation of the percentage of closure of wound over time, offering valuable comprehensions into the efficacy of distinct dressings in fostering the process of wound healing (Amruth *et al*., 2023).

Histopathology Examination

After the study period, humane euthanasia of the rats was conducted using a CO_2 chamber at a flow rate of 3 ml/min for 5-10 minutes. Subsequently, the skin at wound was meticulously and aseptically detached, followed by fixation in a 10% buffered formalin fixative overnight. Hematoxylin and eosin (H and E) staining was performed on sections of the skin samples that were 5 µm thick and placed in paraffin wax. The dyed slides were examined closely under light microscope fitted with a digital camera and pictures of the skin slices were taken. This made it easier to inspect and record the histological alterations in the tissue by the film dressing.

Statistics

Two way analysis of variance was carried out, the significance of hypothesis was tested at 5% level of significance ($p < 0.05$) and marginal means were compared using Turkey's test. Statistical analysis was performed using SAS 9.3.

RESULTS AND DISCUSSION

The fully dissolved nanochitosan- PVA -Glycerol solution was poured onto a tray and left overnight in a hot air oven at 55°C to obtain a transparent nanochitosan film**.** Techniques like scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR) were utilised to characterize the properties of nanochitosan film which was employed for animal studies**.** The test groups exhibited wound closure within the 16-day timeframe, with the NCH-PVA-GLY transparent film dressing (Experimental control-EC) manifesting accelerated healing in contrast to the commercially treated wounds (Positive control-PC) and wound untreated group (Negative control-NC). Figure 1 provides a visual representation of the wound closure rate (WCR) over time for the test groups. WCR measurements were taken on days 3, 7, 12, and 16, revealing a notably higher closure rate in the nanochitosan transparent film dressing-treated group compared to both the control and commercial dressing-treated counterparts. Remarkably, the experimental control resilience throughout the application period exhibited no adherence to the wound. By day 16, nearly complete healing was evident in the experiment control (92.2%) and positive control (87.8%) groups, and negative control (81.1%) as depicted in Table 1 and Figure 2. Maximum wound closure was obtained for the EC incorporated with NCH-PVA-GLY transparent film dressing on 3rd , 7th, 12th,and 16th day respectively when compared to the commercially treated wounds (PC) and wound untreated group (NC). The means were significantly different with each other with $P < 0.05$ (Mostafavinia *et al*., 2021).

Table 1.Two-Way ANOVA of wound closure rates.

Days	Groups and WCR values (%)			Mean
	NC	EC	PC	
0	0	0	0	0^{E}
3	7.78	11.11	8.89	9.260^{D}
7	25.56	31.11	26.66	27.778^{C}
12	51.11	62.22	54.44	55.925^{B}
16	81.11	92.22	87.78	87.037^{A}
Mean	33.1120^{C}	39.3327^{A}	35.5557^{B}	

Mean with different letters on the superscript indicates significance $P < 0.05$

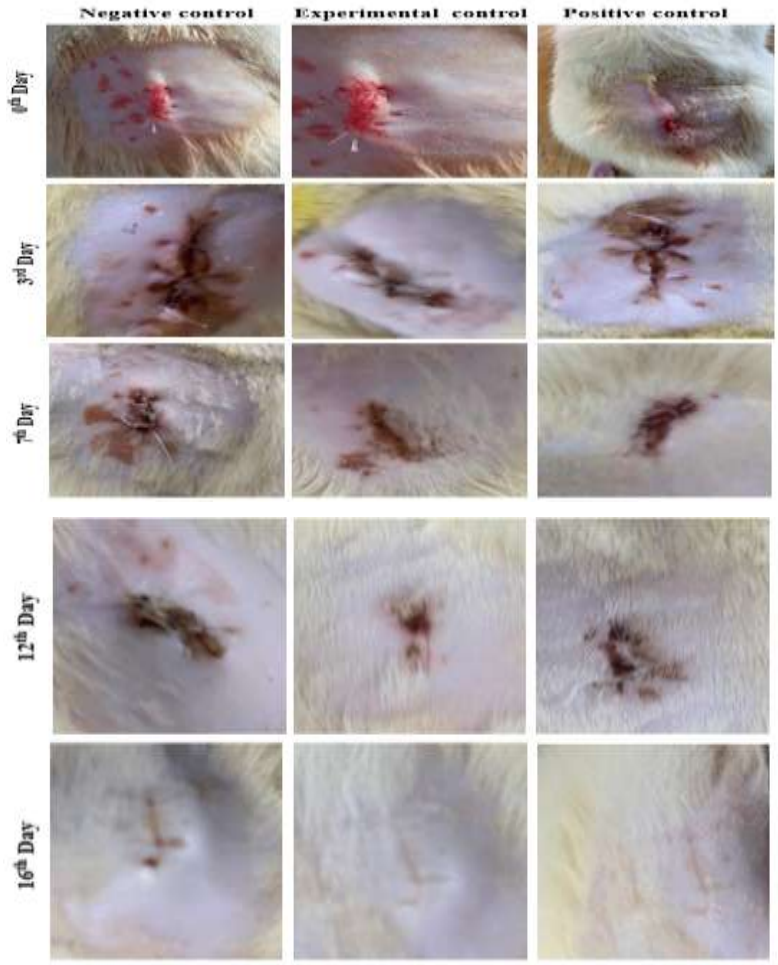

Figure 1. Appearance of the wounds treated with different kinds of wound dressings and wound closure rate.

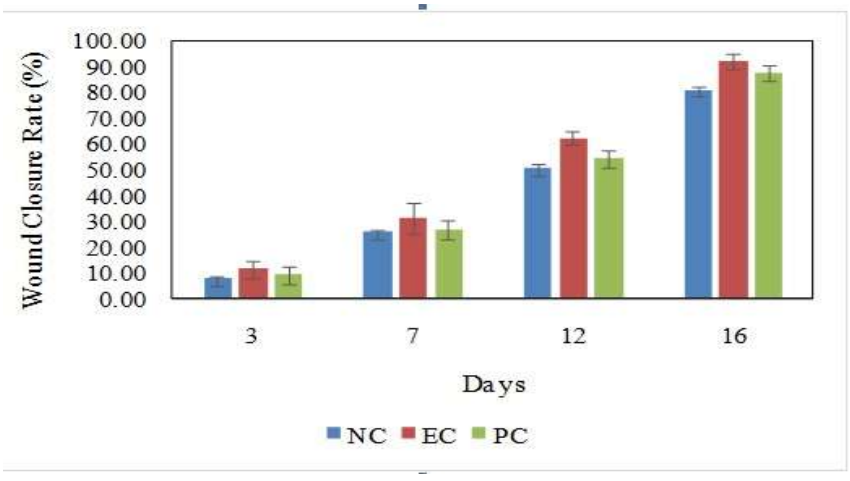

Figure 2. Wound healing rate of three groups over 16 days following treatment and error bars represent the mean of six repeats ± standard deviation.

Notably, wounds subjected to the experimental control displayed superior tissue quality and minimized scarring. In the intricate process of wound healing, chitosan undergoes gradual breakdown, releasing N-acetyl amine (Singh *et al*., 2017). This compound plays a pivotal role in promoting proliferation of fibroblast and orchestrating the systematic deposition of collagen. Additionally the overall healing process was stimulated by the synthesis of natural hyaluronic acid at the wound site (Jayakumar *et al*., 2011; Amruth *et al*., 2023).

The histological assessments provide crucial insights into the healing status. The wound treated with experimental control displayed evident epidermal thickening, with the wound surface exhibiting coverage by new epithelium, as depicted in Figure 3. The presence of pronounced regeneration of skin was noted, featuring well-developed dermal and epidermal layers. In the dermal region, mature fibrous tissue proliferation was discernible. Contrastingly, the negative control group's wound exhibited loose granulation tissue and limited blood capillary development. In the groups treated with the commercial dressing, histological examination revealed inflammatory cell infiltration, and both the epidermis and dermis displayed loosely arranged, immature fibrous tissue formation. Overall, the histological observations indicated positive indications of wound healing in the nanochitosan film dressing-treated group and the healing process was markedly superior in wounds treated with the transparent film dressing (Amruth *et al*., 2023).

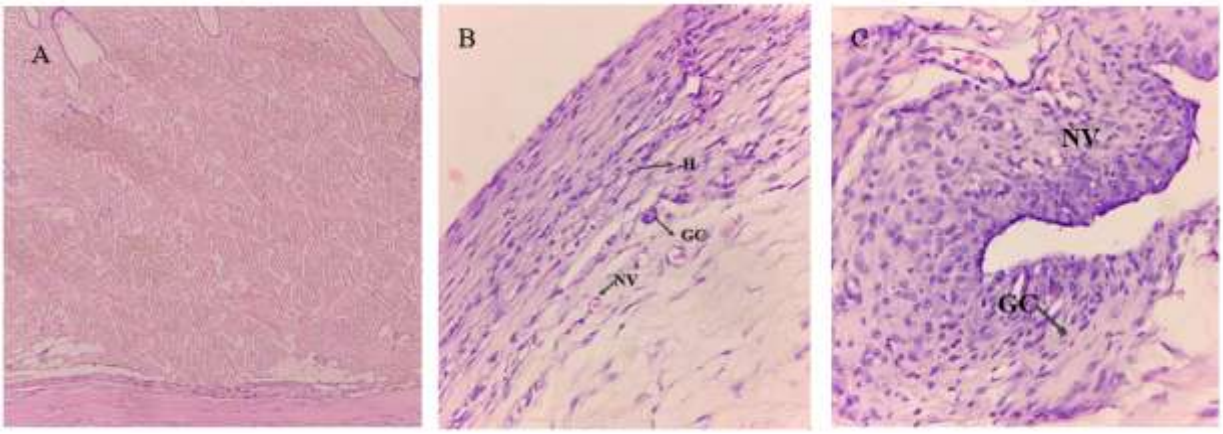

Figure 3. Histopathology observations indicating regeneration potential in experimental group (B) and positive group (C) compared to negative control (A). The markings followed includes, Gc-Giant cells, NV- Neovascularisation, H- Histiocytes.

Table 2. Histopathological findings of Hematoxylin-Eosin Stain.

Histological Observations	Negative control	Experimental control	Positive control
Presence of infolded scaffold	-	Absence	Absence
Presence of cornified layer	Presence	Present	Present
Presence of collagen fibres	Absence	Present	Present
Presence of inflammatory cells	Absence	Presence	Presence
Presence of necrotic cells	Absence	Absence	Absence
Presence of giant cells	Absence	Presence	Presence
Presence of fibrosis	Not found	Less fibrosis	Moderate fibrosis
Presence of neovascularization	Absence	Presence	Absence

Concerning the biodegradability, neither the experimental control nor the positive control exhibited observable degradation. The biocompatibility of the implanted wound dressing material underwent evaluation over a 60-day period. After 28 days, the material was visible and surrounded by a very small amount of lymphocyte inflammation on the histological findings (as evidenced from Figure 4). When any foreign substance is inserted, inflammation is frequently observed and is primarily constituted of lymphocytes, foamy histiocytes, and many multinucleate large cells. These cells are normally present during the acute phase of inflammation (Vande Vord *et al*., 2002). Fibroblast production is a reliable sign of tissue regeneration and the wound-healing process (Dara *et al*., 2021).

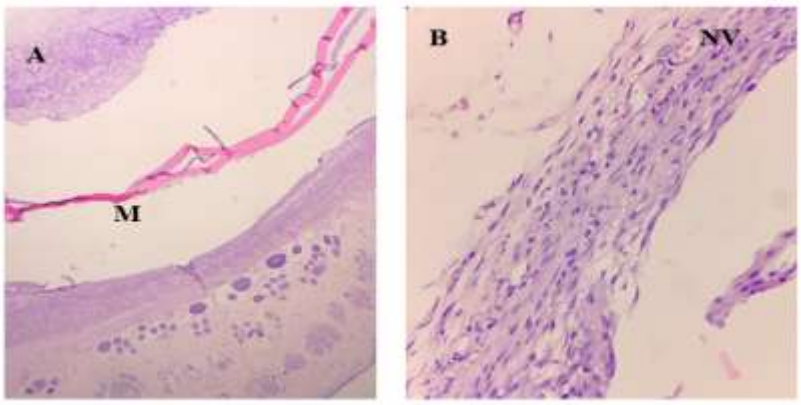

Figure 4. Histopathological section of complete degradation of the material A. 28 days B.60 days .The markings followed includes, M- Presence of material, NV- Neovascularisation.

CONCLUSION

The current experimental investigation into wound healing affirms the NCH-PVA-GLY film dressing's ability to facilitate effective hemostasis, efficient healing, and re-epithelialization of wounds. Histopathological findings indicated that the presence of the dressing stimulates the regeneration of skin cell layers, contributing to the restoration of tissue architecture. The positive outcomes observed in *in vivo* studies on experimental animals underscore the dressing's potential efficacy. Nevertheless, to establish its effectiveness and safety for human patients, further clinical studies are imperative. These future investigations will provide more comprehensive insights, serving as a crucial step toward potential clinical applications of the resultant dressing.

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